Levels of immune regulation in rheumatoid arthritis and in its mouse model, collagen-induced arthritis

PhD thesis

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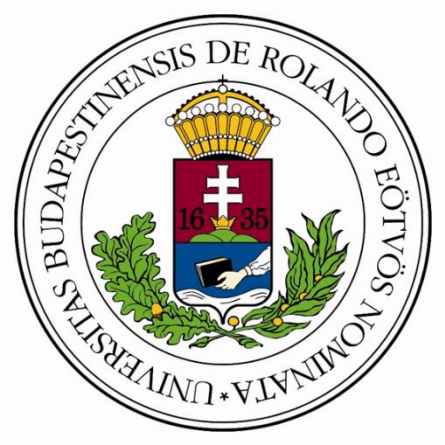

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Introduction

The most important feature of the immune system is that it can differentiate between self and non-self, and responds by either immune tolerance or immune response. If immune tolerance is impaired and the immune system recognizes self-structures e.g. as a consequence of a post-translation protein modification, autoimmunity may arise. Rheumatoid arthritis (RA) is one of the most frequent systemic inflammatory autoimmune disease, primarily affecting the joints of the hands and the legs. Its prevalence worldwide is 0.5 to 1 %. The most efficient and highly specific laboratory diagnostic method known today for RA is the detection of anti-citrullinated protein/peptide antibodies (ACPA) generated as a consequence of post-translational modification of several proteins in patients. An immune response is developed against such modified (citrullinated) patterns foreign to the immune system and auto-antibodies are produced. Citrullination takes place in the body also under physiological conditions, but when rheumatoid arthritis develops – it is presumed – the appearance of modified proteins breaks the immune tolerance [1]. In recent decades, a number of assays have been developed for the detection of ACPAs, and numerous auto-epitopes have been identified. As auto-antibodies are present in patient's blood even several years before the symptoms appear, it is important to improve the sensitivity of diagnostic tests in order to diagnose the disease as early as possible and to set up "risk groups" [2]. Our group established an ELISA system earlier [3], consisting of four citrulline containing peptides based on the sequences of known auto-antigen proteins (citrullinated filaggrin, collagen and vimentin), the diagnostic efficiency of which was compatible with commercially available tests. Although this peptide panel detected ACPA in serum samples with high sensitivity, one of our objectives was to increase the sensitivity of the assay by including further citrulline-peptides into the panel. Moreover, we aimed to compare ACPAs in terms of specificity and affinity.

The course of the disease is fluctuating, with acute and remission phases alternating with each other, unfortunately however, no full recovery can be achieved at patients in the absence of an appropriate therapy. The role of regulatory T and B cells is described as key to the development of remission periods, as they may regulate inflammation by several suppressive mechanisms [4]. Basic research focusing on regulatory cells is very important to better understand the mechanism of the disease, and to reveal pathologic processes. Since it is difficult to reach remission in RA patients and it would take a long time, and it even depends on the individual whether it can occur at all, we turned to the animal model, collagen-induced arthritis (CIA) to study the remission phase and to understand better the mechanism of the disease. We were

particularly interested in B cells producing IL-10, presumably playing a role in the remission phase of collagen-induced arthritis. Our primary objective was to characterize the IL-10 production of marginal zone B cells (MZ B) [5, 6] considered as a potential regulatory B cell (Breg) population based on the data in literature and to identify molecules that are important in their regulation. Besides IL-10 cytokine expression, the T-bet transcription factor [7], was also included in our studies; its role in the differentiation of regulatory T cells has already been described [8-10]. However, its role in B cells is not clarified yet. Our group newly identified the significance of T-bet in B cells, serving as a junction point of the B cell receptor (BCR) and the Toll-like receptor 9 (TLR9) signaling pathways. The simultaneous activation of both pathways synergistically increased the expression of T-bet [11]. Since it is known that these signals (BCR/TLR9) are important inductive factors of regulatory B cell differentiation [6, 12] – and to the analogy that T-bet plays an important role in regulating the suppressive function of a small population of $CD4^+$ regulatory T cells [9] – we were curious to know whether T-bet, activated by the signals through these receptors, plays a role in the IL-10 production of Breg cells, particularly MZ B cells.

Main goals

1. Our goal was to further improve the "4-peptide-panel" ELISA test developed earlier by the group, by including further peptides in the tests with citrulline content (EBNA1/2, α-enolase, fibrine-β), corresponding to sequences from further citrullinated proteins associated with RA, as well as a designed "multi-epitope" peptide.
2. We examined the cross-reactivity of various antibodies recognizing peptides with citrulline content, namely we tested whether an ACPA affinity purified by a given citrullinated peptide can recognize another citrulline containing epitope.
3. There is only a limited amount of data on the affinity of auto-antibodies, especially ACPAs. We wanted to find an answer to the question whether the affinity of anti-citrullinated protein / peptide antibodies contributes to the development of pathological processes.
4. We tracked changes in the expression of IL-10, T-bet, and chemokine receptor CXCR3 regulated by T-bet in MZ B cells in the mouse model of human rheumatoid arthritis, that is, collagen-induced arthritis.
5. We intended to find out whether an in vitro "inflammation" stimuli – activation on BCR/TLR9/IFNγR receptors - would lead to the development of double positive MZ B cells, jointly expressing IL-10 and T-bet molecules.

6. We examined the role of chemokine receptor CXCR3 regulated by T-bet in the life of MZ B cells, whether the cells can migrate towards the receptor's ligand – the chemokine also found in inflamed areas.
7. And finally, we wanted to identify the impact of reactivation on the IL-10 production of MZ B cells pre-activated through BCR/TLR9/IFNγR receptors when placed into a medium which models an "inflammatory environment".

Methods

- identification of antibodies specific to peptides with citrulline content from RA patient's serum samples using an indirect ELISA method
- isolation and cleansing of antibodies specific to peptides with citrulline content from RA patient's serum on an affinity column for citrullinated peptides adsorbed to the solid phase
- affinity examination of antibodies specific to citrullinated peptides using a surface plasmon resonance (SPR) device
- induction of collagen-induced arthritis, tracing the disease
- isolation of mouse spleen B cell sub-populations; in vitro examination of cells
- gene expression analysis with real-time PCR
- detection of cell surface and intracellular molecules with flow cytometry
- measurement of cytokine IL-10 secreted by cells from supernatant cell culture using an ELISA method
- examination of cell migration capability by "trans-well migration assay"
- modelling an "inflammatory environment" by carageenan-induced inflammation model

Results

1. Examination of the diagnostic efficiency of improved citrulline-peptide panel

The peptide ELISA test developed by us earlier ("4-citrulline-peptide-panel", [3]) was further improved ("8-peptid-panel" - filaggrin 5- and 19-mer, vimentin, collagen, EBNA-2, α-enolase, fibrine-β, "multi-epitope") to increase the diagnostic efficiency of the test: its 85% sensitivity value proved to be more efficient compared to approved, commercially available diagnostic tests (approx. 60-80% sensitivity).

2. Examination of the cross-reactions of antibodies specific to citrullinated peptides

We established that an antibody recognizing a given peptide with citrulline content can recognize not only one epitope, but it can react with several citrullinated epitopes originated from different proteins. We analyzed the cross-reactivity of affinity purified ACPAs specific for one citrulline- peptide and we found that the degree of cross-reactivity differs by individuals, meaning that antibodies from each patient may recognize different epitope combinations.

3. Affinity measurement of antibodies specific to citrullinated peptides by SPR device

Our results confirmed that antibodies recognizing peptides with citrulline content (ACPA) have lower affinity compared to antibodies specific to "non-auto-antigens". In order to clarify whether the affinity values of antibodies recognizing citrullinated peptides examined by us (filaggrin, vimentin, "multi-epitope") are associated with the DAS28 index indicating the severity of the disease, we performed a correlation analysis. We found that out of the three peptides examined, in case of filaggrin 19-mer peptide there is an inverse correlation between the dissociation constant (K_D) and the DAS28 index, which means that patients with higher DAS28 values had lower affinity ACPA in their serum.

4. In vivo examination of the IL-10 and T-bet expression of MZ B cells in the induction of collagen-induced arthritis

In order for the in vivo examination of the role of IL-10 and T-bet molecules in MZ B cells in the course if collagen-induced arthritis, we examined the degree of expression of the two molecules in various stages of the disease. We established as a new result that the mRNS level of both IL-10 and the T-bet showed an increase in remission compared to the control

and the acute patient group. And as we expected, changes in the CXCR3 [13] gene expression, regulated by T-bet, also showed significant deviations, similarly to T-bet.

5. *IL-10$^+$/T-bet$^+$ MZ B cell detection following activation on BCR/TLR9/IFNγR receptors*

Potential connections between IL-10 and T-bet were examined by flow cytometry in MZ B cells in vitro, activated through BCR-TLR9-IFNγR (anti-IgM-10μg/ml; CpG-1μM; IFNγ-50ng/ml) receptors. As a result of activation through the three receptors, nearly 100% of MZ B cells expressed the T-bet transcription factor and about 16% of them resulted to be double positive, expressing both the IL-10 and the T-bet molecules. It is important to emphasize that all IL-10$^+$ MZ B cells were T-bet positive as well, so for these signals, a small IL-10 sub-population was managed to be identified within the population of T-bet$^+$ MZ B cells.

6. *In vitro examination of the migration of Tbet$^+$/CXCR3$^+$ Marginal Zone B cells*

Being a member of the T-box transcription family, T-bet regulates the expression of the CXCR3 chemokine receptor at IL-10 producing regulatory T cells, and promotes their migration to inflamed areas [8]. Furthermore, it has also been revealed that it plays an important role in the migration of CXCR3 expressing, antibody producing plasm cells to inflamed areas [14]. In order to clarify the functional relevance of CXCR3 in the life of MZ B cells, we examined the degree of in vitro expression of the two molecules and the migration capability of the cells. Following Anti-IgM, CpG and IFNγ (anti-IgM-10μg/ml/ BCR; CpG-1μM/ TLR9; IFNγ-50ng/ml/ IFNγR) treatment, nearly 70 % of MZ B cells became double positive, expressing both of the T-bet and CXCR3 molecules. And then, after measuring the migration tendency of these cells towards one of the ligands (CXCL9) of the CXCR3 receptor, we found that T-bet and CXCR3 positive cells migrated to a greater extent in the direction of the ligand than negative control cells did.

7. *Examination of IL-10 production in pre-activated MZ B cells after modelling an "inflamed environment"*

Our recent results show that following activation through BCR/TLR9/IFNγR receptors, MZ B cells respond by increased IL-10 production; in addition in vivo, the CIA remission phase witnesses the increased mRNS expression of T-bet, IL-10, and the CXCR3 receptor regulated by T-bet, and we also demonstrated that in vitro, cells can migrate to these signals subject to chemokine gradient control. In order to clarify the biological significance of this,

we set up an experiment system to model an "inflamed environment". According to our hypotheses, if these cells – being pre-activated through BCR/TLR9/IFNγR receptors – are re-activated, perhaps by repeatedly meeting "inflammation stimuli" when migrating to the inflamed area, they can perform their suppressive function even more effectively and respond by more robust IL-10 production. We applied the so-called "air pouch" model [15, 16] – a widely used inflammation model triggered by carrageenan – to model the inflamed environment, acute arthritis. This method is based on the fact that an immunogenic substance (carrageenan) introduced into the hollow produced by air injected under the skin of the animal provokes a reaction very similar to arthritis. We used the fluid isolated from carrageenan-induced pouches to model the inflamed environment. After re-activating pre-activated MZ B cells – that is, having relocated them into the fluid isolated from air pouches, we found that induced T-bet and CXCR3 positive MZ B cells – pre-stimulated through three receptors (BCR-TLR9-IFNγR) – responded by extensive IL-10 production.

Conclusions

On the whole, it can be stated that we were able to use our examinations to increase the efficiency of the test developed for the serological diagnosis of rheumatoid arthritis; and by characterizing the IL-10 production of MZ B cells in collagen-induced arthritis, we got closer to a better understanding of mechanisms controlling B cell development and functions. We further improved the ELISA test – set up earlier by our group and suitable for identifying antibodies recognizing epitopes with citrulline content (ACPA) – by involving citrullinated peptides not yet examined in such combination. The extended ELISA test including eight citrullinated peptides (filaggrin 5 and 19 mer, vimentin, collagen, EBNA-2, α-enolase, fibrin-β, "multi-epitope") slightly exceeded the sensitivity of approved, commercially available diagnostic tests by its 85% sensitivity value, so we managed to increase the diagnostic efficiency of the test. As the anti-citrullinated protein/peptide antibodies of each patient recognize different epitope combinations, the immunological diagnostics of RA can be made more accurate by using a test compiled from heterogeneous peptide sequences. In addition, we proved that the affinity of individual's antibodies recognizing citrullinated epitopes is low (10^{-4}-10^{-7} M/l), they recognize different epitope combinations, they are capable of cross-reaction, and they can recognize more than one epitope; therefore, we believe that patient groups with different ACPA profiles can be

diagnosed more effectively by a diagnostic test containing a mix of heterogeneous peptide sequences, even designed sequences consisting of specific epitopes. We performed a correlation analysis in order to clarify whether the affinity values of antibodies specific to the peptides examined by us (filaggrin, vimentin, "multi-epitope") can be associated with the DAS28 index indicating the severity of the disease. We established that there is an inverse correlation between the K_D measured on citrullinated filaggrin peptide and the DAS28 index, which means that patients with higher DAS28 index values indicating the severity of the disease had lower affinity levels of antibodies specific to citrullinated fillagrin present in their serum. According to hypotheses, the low avidity of antibodies may affect the biological activity of ACPAs and thereby they may lead to the development of a more serious disease [17].

In order to better understand the mechanism of rheumatoid arthritis, we conducted investigations in the animal model of the disease – collagen-induced arthritis, and characterized the IL-10 production capability of MZ B cells. Based on our in vivo and in vitro results, we managed to reveal a new role of the T-bet transcription factor in the life of IL-10 producing MZ B cells. According to our hypothesis, the fact that the expression of IL-10, T-bet and the CXCR3 receptor increases in remission; that in vitro "inflammation" signals trigger the production of T-bet$^+$/IL-10$^+$ and T-bet$^+$/CXCR3$^+$ MZ B cells with a migration tendency; and that they respond to a milieu modelling an inflamed environment by robust IL-10 production, leads us to conclude that the T-bet transcription factor can contribute to the remission of collagen-induced arthritis by supporting the migration of MZ B cells and promoting their more efficient suppressive function. We believe that these results of ours can contribute to a better understanding of the mechanisms controlling the function of regulatory B cells, and finally, to the improvement of therapies to help cure rheumatoid arthritis.

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Publications based on the presented work

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MZ B cells migrate in a T-bet dependent manner and might contribute to the remission of collagen-induced arthritis by the secretion of IL-10; **EUROPEAN JOURNAL OF IMMUNOLOGY** 46:(9) pp. 2239-2246. (2016) *IF: 4.227*

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Other publications

Judit Pozsgay, Eszter Szarka, Krisztina Huber, Fruzsina Babos, Anna Magyar, Ferenc Hudecz, Gabriella Sármay; Synthetic Peptide-Based ELISA and ELISpot Assay for Identifying Autoantibody Epitopes; Peptide Microarrays: Methods and Protocols . Berlin; Heidelberg; New York: **Springer**, 2016. pp. 223-233. (ISBN:978-1-4939-3036-4)

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